AVENIO ctDNA Analysis Kits V2

Reagent Instructions for Use

Version 1.6
Software Version 2.1.0

For Research Use Only. Not for use in diagnostic procedures.

Publication information

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1.0.0	1.0	March 2017	First version.
1.1	1.1.0	January 2018	Updated instructions for software version 1.1.0.
1.2	1.1.0	April 2019	Added important notes and updated quantities in kits.
1.3	2.0.0	October 2020	Updated Illumina sequencing kit and catalog number in consumables list purchased from other vendors, and made minor corrections
1.4	2.0.0	October 2021	Updated quantity of Bead Wash Buffer (2.5X)
1.5	2.0.0	November 2021	Correction to catalog number for NextSeq 500/550 High Output Kit v2.5 kit (300 cycles)
1.6	2.1.0	July 2023	The AVENIO ctDNA Analysis kits were updated to V2 kits to align with both manufacturing/operational changes and in compliance with Registration, Evaluation, Authorization and Restriction of Chemicals (REACH). Clarified information and instructions, and added the TapeStation and LabChip as additional platforms for sample analysis.

Edition notice

This publication is intended for AVENIO ctDNA Analysis Kits V2.

Every effort has been made to ensure that all the information contained in this publication is correct at the time of publishing. However, the manufacturer of this product may need to update the publication information as output of product surveillance activities, leading to a new version of this publication.

Where to find information

This *AVENIO ctDNA Analysis Kits V2 Reagent Instructions for Use* contains information about using the reagents, organized according to the normal operation workflow.

The *AVENIO Oncology Analysis Software User Guide* contains information about using the analysis software, organized according to the normal operation workflow.

The *AVENIO Oncology Analysis Software Admin Guide* contains information about setting up and managing the analysis software for users.

General attention

To avoid incorrect results, ensure that you are familiar with the instructions before you use the product.

- Pay particular attention to all important notes.
- Always follow the instructions in this publication.
- Do not use the reagents in a way that is not described in this publication.
- Store all publications in a safe and easily retrievable place.

Screenshots

The screenshots in this publication have been added exclusively for illustration purposes. Configurable and variable data, such as tests, results, or path names visible therein must not be used for laboratory purposes.

Warranty for the reagent

Roche warrants that this product will meet the specifications stated in the labeling when used in accordance with such labeling and will be free from defects in material and workmanship until the expiration date printed on the label.

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Feedback

Every effort has been made to ensure that this publication fulfills the intended use. All feedback on any aspect of this publication is welcome and is considered during updates. Contact your Roche representative, should you have any such feedback.

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Technical support

For product-related technical documents or to contact Roche Technical Support, go to *sequencing.roche.com/support*.

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Preface

Regulatory Disclaimer

For Research Use Only. Not for use in diagnostic procedures.

AVENIO ctDNA Analysis Kits V2

The AVENIO ctDNA Analysis Kits V2 are a portfolio of three next-generation sequencing liquid biopsy assays designed to help researchers understand the genomic complexity of aberrations in solid tumors. The kits are for research use only, not for use in diagnostic procedures. The AVENIO ctDNA Analysis Kits V2 provide an end-to-end tumor profiling and monitoring solution, from cell-free DNA (cfDNA) extraction to analysis and variant reporting.

Additonal AVENIO products

Except where the context clearly indicated otherwise, the following product names and descriptors are used.

Product name	Descriptor
AVENIO ctDNA Analysis Software	Analysis software

🔍 An on-premise Oncology Analysis Server (OAS) allows laboratory professionals to produce secondary analysis variant calls locally, review Quality Control (QC) metrics, and run analytical concordance reports. The OAS runs the AVENIO Oncology Analysis Software and is necessary to provide analysis setup and reporting functionality for the entire AVENIO family of next-generation oncology assays. The OAS is available for purchase from Roche.

Symbols and abbreviations

Symbols used in the publication

Symbol	Explanation
⚠	**Safety alert note**: The safety alert symbol is used to alert you to potential physical injury hazards. To avoid possible injury or death, comply with all safety messages that follow this symbol.
❗	**Important note:** Highlights information that is critical for optimal performance of the system. May also indicate that loss of data or invalid data could occur if the precautions or instructions are not observed.
🔍	**Information note**: Identifies items of general interest and additional information about the topic or procedure being described.

Symbols used on product

Symbol	Explanation
	**Corrosion** ■ Skin corrosion/burns ■ Eye damage ■ Corrosive to metals
	**Exclamation Mark** ■ Irritant (eye and skin) ■ Skin sensitizer ■ Acute toxicity ■ Narcotic effects ■ Respiratory tract irritant ■ Hazardous to ozone layer (non-mandatory)
	**Health Hazard** ■ Carcinogen ■ Mutagenicity ■ Reproductive toxicity ■ Respiratory sensitizer ■ Target organ toxicity ■ Aspiration toxicity
	**Skull and Crossbones** ■ Acute toxicity (fatal or toxic)

Symbol	Explanation
🟡	A yellow top-dot sticker is used on vial caps, when possible, for reagents exclusive to the AVENIO Tumor Tissue Analysis Kits V2 workflow.
⚪	A white top-dot sticker is used on vial caps, when possible, for reagents exclusive to the AVENIO ctDNA Analysis Kits V2 workflow.
🟢	A Green top-dot sticker is used on the AVENIO Post-Hybridization Kit V2. The AVENIO Post-Hybridization Kit V2 is the only sub-kit that is jointly used by both the AVENIO Tumor Tissue and the AVENIO ctDNA workflows.

Abbreviations and acronyms

The following abbreviations and acronyms are used.

Abbreviation/acronym	Definition
bp	Base pair
cfDNA	Cell free DNA
CNV	Copy Number Variant
CRC	Colorectal Cancer
CT	Collection Tube
ctDNA	Circulating tumor DNA
DNA PBB	DNA Paraffin Binding Buffer
EB	Elution Buffer
EDTA	Ethylenediaminetetraacetic Acid
EO	Enhancing Oligo
FT	Filter Tube
HPEA	High Pure Extender Assembly
Indel	Insertion and deletion
NCCN	National Comprehensive Cancer Network
NSCLC	Non-Small Cell Lung Cancer
PCR	Polymerase Chain Reaction
PK	Proteinase K
QC	Quality Control
REACH	Registration, Evaluation, Authorization and Restriction of Chemicals
SA	Sample Adapter
SNV	Single Nucleotide Variant
WB I	Wash Buffer I
WB II	Wash Buffer II
∞	Hold

What is new in publication version 1.6

- The AVENIO ctDNA Analysis Kits were updated to V2 Kits to satisfy Registration, Evaluation, Authorization and Restriction of Chemicals (REACH) compliance requirements. The REACH compliant reagents are designed to provide optimal results with the new workflow conditions, therefore, using the previous workflow with V2 reagents will lead to suboptimal assay performance, delayed results and potential loss of sample, resulting in poor libraries and poor sequencing results, requiring the user to perform the workflow again.
- The AVENIO ctDNA Analysis Kits V2 were updated to align with operational requirements and changes in manufacturing.
- The Hybridization workflow was optimized due to the newly launched REACH-compliant reagent. The volumes of the components changed for the Hybridization Master Mix to obtain optimal concentrations within 43 µL per reaction. The concentration of the panels changed due to manufacturing changes, and thus 4 µL is required per reaction. In addition, the incubation temperature for the Hybridization reaction, Hybridization Wash 1 and Stringent Wash Buffer were changed from 47°C to 60°C.
- Safety alert notes, important notes and information notes were added and updated throughout the document.
- The 4200 TapeStation and LabChip GX Touch HT Nucleic Acid Analyzer were added as alternative platforms for library quality assessment. In addition, NextSeq 550Dx was added as an alternative sequencing analyzer.

Protocol information and safety

- Apply good laboratory practices, such as wearing appropriate personal protective equipment when handing biological material.
- Wear gloves and take precautions to avoid sample contamination.
- Change gloves when opening and closing strip tubes to minimize cross-contamination.
- Take precautions to avoid sample cross contamination, especially prior to the first PCR reaction, which is the step when unique sample barcodes are tagged to the DNA.
- It is recommended to perform cfDNA isolation and pre-PCR procedures in an amplicon-free area, preferably, a separate lab, to minimize contamination. Clean work area thoroughly before and after lab procedures.
- Unless otherwise specified, all mixing steps are listed as "mix thoroughly" and indicate that the sample should be mixed by either vortexing for 5 seconds or pipetting up and down 10 times.
- If liquid has collected in a tube's cap after mixing, gently tap or briefly spin the sample to collect the liquid into the tube's bottom, ensuring that the mixture remains homogeneous before progressing to the next step.
- Properly label tubes at all times to prevent sample mix-up.
- Check each reagent name and expiration date before use.
- Prepare 80% ethanol fresh on the day of usage using 100% ethanol and nuclease-free water, PCR grade.
- It it recommended to use a multi-channel pipettor to ensure sample consistency.
- Thaw components that contain enzymes on ice, and keep on ice during procedure. All other components, including primer pools, can be thawed at room temperature. Gently vortex and centrifuge before use.
- When provided, follow the recommended heated lid temperature for the thermocycler incubations. Otherwise, it is recommended to set the lid to 10°C above the block temperature.
- Be careful when removing tubes from the thermocycler as the caps can pop open due to the high incubation temperature.
- Perform all centrifugations at room temperature (+15°C to +25°C) unless instructed otherwise.
- Laboratory temperature should be maintained at room temperature (+20°C to +25°C) to avoid freezing of Hybridization Buffer 2 V2.
- Safe stopping points of the assay are after the DNA extraction, after the PCR cleanup of the pre-capture library, and the final enriched library. Consult the procedures for the storage conditions of the stopping points.
- Ensure hazardous waste is properly disposed.
- Hybridization Buffer I and PCR Reaction Mix (2X) contain tetramethylammonium chloride. Practice safe laboratory practices when handling these reagents.
- The QC measurements by the Qubit dsDNA HS quantification assays can also be performed using the Qubit 1X dsDNA HS Assay Kit according to manufacturer's instructions.
- The QC measurements performed by the Agilent 2100 Bioanalyzer High Sensitivity DNA trace analysis can be substituted with the Agilent D1000 / High Sensitivity D1000 ScreenTape assay (on a 4200 TapeStation) or a LabChip NGS 3K reagent kit with HT DNA X-Mark Chip on a LabChip GX Touch HT Nucleic Acid Analyzer.

Required equipment, labware, and consumables

The protocol is designed for use with the specified equipment, labware, and consumables described below. You assume full responsibility when using equipment, labware and consumables described below.

Laboratory equipment

The catalog numbers referenced in the following table may be United States catalog numbers.

Equipment	Supplier	Catalog number
96-well thermocycler with programmable heated lid capable of 50°C, 85°C, and 105°C (Recommended: Veriti Dx 96-well Thermal Cycler, 0.2 mL, ThermoFisher Scientific, catalog number 4452300)	Multiple vendors	
0.2 mL PCR Strip Magnetic Separator	Permagen Labware	MSR812
DynaMag-2 Magnet	ThermoFisher Scientific	12321D
Serological pipettors (Capable of 2 to 10 mL volumes)	Multiple vendors	
Multi-channel pipettes (8 channel each, capable of 2 to 20 µL and 20 to 200 µL volumes)	Multiple vendors	
Single-channel pipettes (Capable of 0.5 to 2 µL, 2 to 20 µL, 20 to 200 µL, and 200 to 1000 µL volumes)	Multiple vendors	
Qubit 3.0 or Qubit 4 Fluorometer	ThermoFisher Scientific	Q33216, Q33226
2100 Bioanalyzer or 4200 TapeStation System	Agilent Technologies	G2938C, G2940CA, G2943CA G2991BA
LabChip GX Touch HT Nucleic Acid Analyzer	PerkinElmer	CLS137031
Illumina NextSeq 500, NextSeq 550 or NextSeq 550Dx	Illumina	SY-415-1001, SY-415-1002, 20005715
Tabletop swing-bucket centrifuge (Capable of 1800 x g and 3270 x g; with 15 mL and 50 mL volume conical tube compatible swinging bucket rotor)	Multiple vendors	
Tabletop centrifuge (Capable of 8000 x g and 16000 to 20000 x g; for 1.5 and 2 mL microcentrifuge tubes)	Multiple vendors	
Tabletop micro/mini centrifuge (0.2 mL PCR strip tube compatible rotor)	Multiple vendors	
Vortex Mixer	Multiple vendors	

Reagents and consumables available from Roche Diagnostics

Three AVENIO ctDNA Analysis Kits V2 are available that reflect three distinct panel options. Each AVENIO ctDNA Analysis Kit V2 contains reagents for end-to-end processing of cfDNA samples, from plasma extraction to an enriched library ready for sequencing.

Orderable kit name	Orderable kit number	Included sub-kit name
AVENIO ctDNA Targeted Kit V2	09733736001	AVENIO cfDNA Isolation Kit V2
		AVENIO ctDNA Library Prep Kit V2
		AVENIO ctDNA Enrichment Kit V2
		AVENIO ctDNA Targeted Panel V2
		AVENIO Post-Hybridization Kit V2
AVENIO ctDNA Expanded Kit V2	09733779001	AVENIO cfDNA Isolation Kit V2
		AVENIO ctDNA Library Prep Kit V2
		AVENIO ctDNA Enrichment Kit V2
		AVENIO ctDNA Expanded Panel V2
		AVENIO Post-Hybridization Kit V2

Orderable kit name	Orderable kit number	Included sub-kit name
AVENIO ctDNA Surveillance Kit V2	09733817001	AVENIO cfDNA Isolation Kit V2
		AVENIO ctDNA Library Prep Kit V2
		AVENIO ctDNA Enrichment Kit V2
		AVENIO ctDNA Surveillance Panel V2
		AVENIO Post-Hybridization Kit V2

For shipping and storage information, see *Appendix A*.

Cleanup Beads V2 are shipped frozen but should be stored at 2°C to 8°C. They are located within the AVENIO ctDNA Library Prep Kit V2 and the AVENIO ctDNA Enrichment Kit V2. Upon shipment arrival, remove the vials from both kit boxes, and transfer the Cleanup Beads V2 to 2°C to 8°C storage.

Reagents and consumables purchased from other vendors

The catalog numbers referenced in the following table may be United States catalog numbers.

Component	Supplier	Catalog number
0.2 mL 8-strip PCR tubes (required tubes ordered from one of these two suppliers)	Starlab International GmbH or USA Scientific (Western hemisphere)	1402-3700 1402-4700
1.5 mL or 2.0 mL microcentrifuge tubes (low-bind tubes recommended)	Multiple vendors	
5.0 mL microcentrifuge tubes (low-bind tubes recommended)	Multiple vendors	
15 mL or 50 mL polypropylene conical tubes	Multiple vendors	
Sterile pipetting reservoirs	Multiple vendors	
Low-retention pipette tips	Multiple vendors	
5 mL or 10 mL volume serological pipettes	Multiple vendors	
Nuclease-free Water, PCR grade (not DEPC treated)	Multiple vendors	
Ethanol, 200 proof (absolute), molecular biology grade	Multiple vendors	
Isopropanol, molecular biology grade	Multiple vendors	
Qubit dsDNA HS Assay Kit or Qubit 1X dsDNA HS Assay Kit	ThermoFisher Scientific	Q32851 (100 assays) Q32854 (200 assays) Q33230 (100 assays) Q33231 (500 assays)
Qubit Assay Tubes	ThermoFisher Scientific	Q32856
Agilent High Sensitivity DNA Kit (for 2100 Bioanalyzer)	Agilent Technologies	5067-4626
Agilent High Sensitivity D1000 ScreenTape assay (for 4200 TapeStation)	Agilent Technologies	5067-5584 5067-5585 5067-5587
DNA NGS 3K Reagent Kit (for LabChip)	PerkinElmer	CLS960013
HT DNA X-Mark Chip (for LabChip)	PerkinElmer	CLS144006
96-well or 384-well skirted plate (for LabChip)	Multiple vendors	
Dynabeads M-270 Streptavidin	ThermoFisher Scientific	65305
1.0 M NaOH, molecular biology grade (for sequencing)	Multiple vendors	
200 mM Tris-HCl, pH 7.0, molecular biology grade (for sequencing)	Multiple vendors	
PhiX Control V3	Illumina	FC-110-3001
NextSeq 500/550 High Output v2.5 kit (300 cycles)	Illumina	20024908
NextSeq 550Dx High Output Reagent Kit v2.5 (300 cycles) IVD	Illumina	2002881

Chapter 1. Getting started

This guide describes the use of the AVENIO ctDNA Analysis Kits V2 to prepare libraries from cell free DNA (cfDNA) isolated from plasma to identify aberrations such as SNVs (single nucleotide variants), Indels (insertions and deletions), CNVs (copy number variants), as well as fusions from solid tumors. The AVENIO ctDNA Analysis Kits V2 provide reagents to isolate cfDNA from plasma, prepare sequencing libraries, and enrich selected regions of interest using one of three optimized hybridization panels (AVENIO ctDNA Targeted V2, AVENIO ctDNA Expanded V2, or AVENIO ctDNA Surveillance V2 Panels). Specifically, this guide provides a protocol for the workflow outlined below to prepare enriched libraries of ctDNA ready to be sequenced on the third-party Illumina NextSeq 500/550 sequencer and NextSeq 550Dx in RUO Mode. The AVENIO ctDNA Analysis Kits V2 solution also includes analysis software, which is described in the *AVENIO Oncology Analysis Software User Guide*.

About AVENIO ctDNA Analysis Kits V2

The AVENIO ctDNA Analysis Kits V2 provide customers with an optimized workflow to deliver information on detected cancer variants within a plasma sample. These tests utilize hybrid capture target enrichment techniques combined with innovative bioinformatic workflows to deliver a comprehensive genomic profile across four mutation classes: SNVs, Indels, CNVs, and fusions. The AVENIO ctDNA Analysis Kits V2 combine exceptional sensitivity and specificity with the convenience and efficiency that is achieved through liquid biopsies.

- The AVENIO ctDNA Targeted Kit V2 is a 17 gene pan-cancer assay for identifying and characterizing mutations in a wide variety of solid tumors. This Targeted Kit includes genes for NSCLC, CRC, and other cancer types aligned with NCCN guidelines.
- The AVENIO ctDNA Expanded Kit V2 is a 77 gene pan-cancer assay for identifying and characterizing genes associated with solid tumors. The Expanded Kit is designed to help researchers profile mutations in genes relevant for clinical trial research. This pan-cancer panel focuses on NSCLC and CRC and other cancer types, and includes variants aligned with guidelines.
- The AVENIO ctDNA Surveillance Kit V2 has been designed to enable monitoring applications in order to reveal how cancers evolve over time. Researchers can use the Surveillance Kit to identify, profile, and monitor mutations in 197 genes, across 471 hypermutated regions. This panel has been optimized for use in NSCLC and CRC.

The AVENIO ctDNA Analysis Kits V2 include the reagents, analysis software, and bioinformatics necessary to uncover genomic aberrations in solid tumors. An Oncology Analysis Server to run the bioinformatic pipeline is required and available from Roche.

Workflow

The AVENIO ctDNA Analysis Kits V2 workflow involves the following high-level steps and designated kits:

1. Isolate cfDNA from plasma
    - AVENIO cfDNA Isolation Kit V2
2. Prepare sequencing libraries
    - AVENIO ctDNA Library Prep Kit V2
3. Perform the enrichment protocol
    - AVENIO ctDNA Enrichment Kit V2
    - AVENIO ctDNA Panels V2 (product-specific panel includes one of the following)
        - AVENIO ctDNA Targeted Panel V2
        - AVENIO ctDNA Expanded Panel V2
        - AVENIO ctDNA Surveillance Panel V2
    - AVENIO Post-Hybridization Kit V2
4. DNA sequencing

The following table details the approximate processing time of each step of the AVENIO ctDNA Analysis Kits V2 workflow.

Step	Processing time
Isolate cfDNA from plasma	4 hours
	Safe stopping point
Prepare DNA for adapter ligation and initiate adapter ligation	2 hours
	Overnight ligation (16-18 hours)
Perform post-ligation cleanup	1 hour
PCR amplify ligated sample and cleanup	1.5 hours
	Safe stopping point
Hybridization of the sample	2 hours
	Overnight Incubation (16-18 hours)
Bind samples to streptavidin beads	1 hour
Perform post-hybridization washes	2 hours
PCR amplify enriched samples and cleanup	2 hours
	Safe stopping point
Final library quantification and pooling for sequencing	2.5 hours

Chapter 2. Isolating cfDNA from plasma

This section of the protocol uses the AVENIO cfDNA Isolation Kit V2 and provides instructions to isolate cfDNA from plasma using an efficient filter tube system. It yields quality cfDNA that is used as input for the rest of the sequencing library preparation workflow in *Chapter 3*.

Quality cfDNA requires proper blood collection methods and careful handling of plasma samples. The user is responsible for ensuring that the selected collection tube is used according to its manufacturer's protocol. Ensure that the blood collection tube contains the anticoagulant EDTA. The anticoagulant heparin should not be used as it will interfere with the library preparation. To minimize cellular lysis, which releases high molecular weight genomic DNA that can interfere with library generation, separate and collect the plasma soon after blood collection (typically within 2 hours of blood collection, but dependent on the tube used). If plasma is not immediately used for the cfDNA isolation procedures, it should be stored frozen at -80°C.

It is critical that the plasma layer is collected with minimal leukocyte contamination. Plasma should be transferred to a new tube without disturbing the buffy coat since leukocytes contain large molecular weight genomic DNA that will reduce the efficiency of sequencing library generation.

It is recommended to use 4 mL of plasma for the AVENIO cfDNA Isolation Kit V2; however, if 4 mL of plasma is unavailable, the kit is able to process 2 to 5 mL of plasma using the exact reagent volumes as listed in the protocol. Enter the exact plasma volume processed in the analysis software.

Up to 16 plasma samples can be processed at a time.

Preparing the cfDNA isolation reagents

When using the kit for the first time, prepare working reagents as follows:

Reagents	Reconstitution/preparation
Proteinase K	Reconstitute Proteinase K by adding 4.5 mL of sterile water to the vial using a sterile, disposable 5 mL serological pipette. Mix by inverting the vial 5 to 10 times. 500 μL is required per specimen. For excess reconstituted Proteinase K, aliquot 1100 μL into 1.5 mL microcentrifuge tubes and store at -20°C for up to 30 days or until the expiration date, whichever comes first.
Wash Buffer I	Add 15 mL of absolute ethanol to the bottle of Wash Buffer I. Mix by inverting the bottle 5 to 10 times. Note on the bottle that ethanol is added and the date. Store working solution at 15 to 30°C for up to 90 days or until the expiration date, whichever comes first.
Wash Buffer II	Add 50 mL of absolute ethanol to the bottle of Wash Buffer II. Mix by inverting the bottle 5 to 10 times. Note on the bottle that ethanol is added and the date. Store working solution at 15 to 30°C for up to 90 days or until the expiration date, whichever comes first.

All solutions stored at 15°C to 30°C should be clear. If precipitate is present in any reagent, warm the solution to 37°C until the precipitate dissolves.

Isolating cfDNA from plasma

To isolate cfDNA from plasma

1. Label one 15 mL conical tube for each sample being processed. Thaw plasma sample, and spin at 1800 x g for 5 minutes to remove precipitates and debris.
2. Using a serological pipette, carefully transfer 4 mL of plasma to a labeled 15 mL conical tube taking care to not disturb the bottom precipitate, if present. If 4 mL of plasma is not available, the isolation procedure can accommodate 2 to 5 mL plasma input using the same reagent volumes noted in the protocol.

> Avoid transferring cell debris from the bottom of the tube as this may introduce large molecular weight genomic DNA into the final elution, which may reduce assay performance.

3. Note the volume of plasma used for each sample. This volume will be used for data analysis.

> This volume is required for the AVENIO ctDNA Analysis Software to compute the *No. of Mutant Molecules per mL* in the Sample Report.

4. Add 500 µL of reconstituted Proteinase K to each plasma sample.
5. Mix by inverting the tubes 3 to 5 times, and incubate at room temperature (15°C to 30°C) for 5 minutes.
6. Add 4 mL of DNA PBB V2 to each plasma sample.
7. Mix by inverting the tubes 3 to 5 times, and incubate at room temperature for 30 minutes.
8. Add 1000 µL isopropanol, and mix by inverting the tubes 3 to 5 times.
9. Transfer the mixture to an appropriately labeled High Pure Extender Assembly (HPEA) Unit. The HPEA Unit is depicted in *Figure 1* and comes pre-assembled as a 50 ml Polypropylene Tube containing a High Pure Extender to hold the plasma, and the Filter Tube to bind the cfDNA.

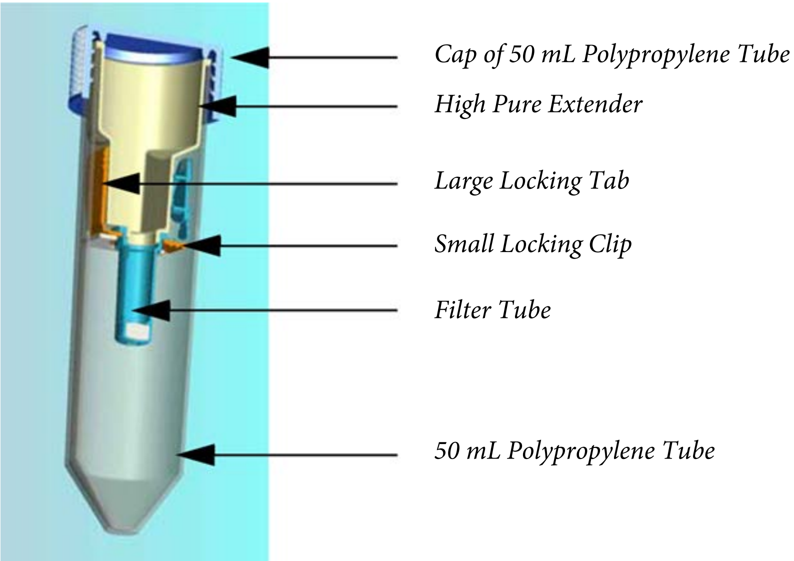

Figure 1. High Pure Extender Assembly (HPEA)

10. Replace the cap, and centrifuge the HPEA unit at 3270 x g for 5 minutes.
11. Remove the High Pure Extender (with attached Filter Tube) from the 50 mL Polypropylene Tube, and place it into a Collection Tube on a tube rack holder. Remove the Large Locking Tab by twisting and pulling it away from the assembly.
12. Remove the Small Locking Clip from underneath the Filter Tube's cap, which became free when the Large Locking Tab was pulled away in the previous step. (If necessary, ensure the Small Locking Clip has broken free, and pull it away.)

13. Snap off the Filter Tube from the High Pure Extender by firmly tilting the Filter Tube/Collection Tube away from the High Pure Extender until the Filter Tube breaks free.
14. Keep the Filter Tube in the Collection Tube. Label the Filter Tube appropriately. The Filter Tube contains the cfDNA.
15. Properly discard as biohazardous waste the 50 mL tube that contains the plasma flow-through.

Immediately proceed to *Washing the cfDNA* to prevent the Filter Tube from drying out.

Washing the cfDNA

To wash the cfDNA

1. Add 500 µL working Wash Buffer I to each Filter Tube to wash the cfDNA. Centrifuge at 8000 x g for 1 minute.

Perform all wash spins on a benchtop microcentrifuge.

2. Place the Filter Tube on a new Collection Tube, and discard the old Collection Tube.
3. Add 500 µL working Wash Buffer II to each Filter Tube to wash the cfDNA.
4. Centrifuge at 8000 x g for 1 minute.
5. Place the Filter Tube on a new Collection Tube, and discard the old Collection Tube.
6. Centrifuge at 16000 to 20000 x g for 1 minute to dry the filter membrane.
7. Place the Filter Tube onto a new, pre-labeled 1.5 mL microcentrifuge tube.
8. Add 65 µL of DNA Elution Buffer onto the center of the Filter Tube membrane without touching the membrane.
9. Incubate for 5 minutes at room temperature.
10. Elute the DNA by centrifuging the Filter Tube and microcentrifuge tube for 8000 x g for 1 minute.
11. Carefully transfer the top 60 µL eluate to a new 1.5 mL microcentrifuge tube without disrupting any debris pellet that may have formed at the bottom of the tube. The pellet may not be visible.
12. Close the cap of the microcentrifuge tube, which contains the cfDNA.
13. Immediately proceed to the next Quality Control (QC) steps, or store the cfDNA sample in the following conditions:

Overnight storage temperature at 2°C to 8°C.

Long term storage temperature at -15°C to -25°C.

Checking the quality of plasma-extracted cfDNA

To check the quality of plasma-extracted cfDNA

1. Quantify the concentration of samples using the Qubit dsDNA HS Assay Kit according to the manufacturer's instructions.

2. Assess DNA quality by diluting samples (if necessary) to a recommended concentration for analysis using Agilent High Sensitivity DNA Kit. Analyze the samples using an Agilent High Sensitivity DNA Assay on a 2100 Bioanalyzer following the manufacturer's instructions. Quality cfDNA should be free of high-molecular weight genomic DNA (see *Appendix B*).

Alternatively, an Agilent High Sensitivity D1000 ScreenTape assay on a 4200 TapeStation or a LabChip NGS 3K reagent kit with HT DNA X-Mark Chip on a LabChip GX Touch HT can be used.

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3. Calculate and record the *Isolated DNA Mass*. This represents the mass of cfDNA isolated from the volume of plasma used in the assay. Assuming 65 µL of DNA Elution Buffer (EB) was used to elute the final cfDNA, use the following formula:

$$\text{Isolated DNA Mass (ng)} = \text{Concentration of isolated cfDNA (ng/µL)} \times 65\ \text{µL}$$

Isolated DNA Mass is required for the AVENIO Oncology Analysis Software to compute the *No. of Mutant Molecules per mL* of plasma in the Sample Report.

Chapter 3. Preparing sequencing libraries

This section of the protocol uses the AVENIO ctDNA Library Prep Kit V2 to prepare sequencing libraries with unique sample IDs. Each sample will be ligated to a unique Sample Adapter, which enables the multiplexed sequencing of up to 16 samples per sequencing run.

Use 50 ng of cfDNA, when available, to maximize mutation detection sensitivity. The number of unique DNA molecules sequenced is directly correlated to the number of input DNA molecules into the assay. However, when 50 ng is unavailable, use all of the available cfDNA, down to 10 ng. The library preparation protocol supports 10 ng to 50 ng cfDNA input ranges. A maximum of 50 µL can be input into library preparation; therefore, adjust the cfDNA concentrations accordingly.

The recommended DNA input amount for the AVENIO ctDNA Analysis Kit V2 is 50 ng for maximum sensitivity and specificity. This test has been developed for 10 ng to 50 ng of input DNA. Greater than 50 ng of cfDNA may require a greater number of sequencing reads per sample.

To ensure that quality cfDNA is used to prepare sequencing libraries, it is recommended to use the AVENIO cfDNA Isolation Kit V2, following the instructions in *Chapter 2*. However, preparation of sequencing libraries can also be performed with cfDNA isolated using other methods. Performance is not guaranteed when using other sources of cfDNA, and the quality of the cfDNA should be checked before preparing libraries (see *Appendix B*).

Although 16 samples can be processed and multiplexed together with high sensitivity, the performance of the assay may be further enhanced with greater sequencing depth, which is achieved by multiplexing fewer samples.

For all master mixes, prepare 10% overage.

The AVENIO ctDNA Analysis Kits V2 can also process cell line and engineered DNA samples. These types of samples can serve as process and performance controls. To process these samples, the DNA must be sheared/fragmented to approximately 200 bp prior to input into the library preparation workflow. Fragmentation protocol is not provided in this guide and is for the user to determine. In general, enzymatic fragmentation yields greater ligation efficiency than mechanically sheared DNA.

Avoid multiplexing cell line or engineered DNA samples with actual biological ctDNA samples on the same sequencing run. Cell lines and engineered DNA may contain poorly-characterized variants and may harbor abnormally high mutant allele fraction, which may interfere with the sequencing of biological cfDNA samples.

Each sample must receive a unique Sample Adapter. Keep track of each sample to ensure that the proper Sample Adapter number is entered in the analysis software.

Do not mix AVENIO and non-AVENIO assays on the same NextSeq run.

Preparing the cfDNA sample for ligation

To prepare the cfDNA sample for ligation

1. Add 50 ng of cfDNA into a 0.2 mL strip tube, up to a maximum of 50 µL. If 50 ng is unavailable, add the maximum amount down to 10 ng, to a maximum of 50 µL.
2. Add nuclease-free water, PCR grade, to the cfDNA to bring the total volume to 50 µL.
3. Prepare the DNA Preparation Master Mix:

DNA Preparation Master Mix	
Reagent	**Volume per reaction**
DNA Preparation Buffer	7 µL
DNA Preparation Enzyme V2	3 µL

4. Add 10 µL of DNA Preparation Master Mix to each sample.
5. Mix by pipetting or vortexing, and briefly spin down the tubes to collect the liquid at the bottom.
6. Incubate on a thermocycler using the following program:

Temperature	Duration
20°C	30 minutes
65°C	30 minutes
4°C	∞

> Set the thermocycler lid to 85°C and volume to 60 µL.

7. Proceed immediately to *Performing Sample Adapter ligation*.

Performing Sample Adapter ligation

To perform Sample Adapter ligation

1. Prepare the Ligation Master Mix:

Ligation Master Mix	
Reagent	**Volume per reaction**
Ligation Buffer	30 µL
DNA Ligase	10 µL

> The Ligation Master Mix is highly viscous. Extra care is needed to mix the master mix well and to aliquot the right amount to each tube and mix well.

2. Add 10 µL of a unique Sample Adapter to each sample.

> **Each sample must receive a unique Sample Adapter. Record the Sample Adapter number (SA#) used, as the Enhancing Oligo number (EO#) must match the SA#. In addition, the SA# will be entered into the AVENIO ctDNA Analysis Software.**

3. Mix by pipetting or vortexing, and briefly spin down the tubes to collect the liquid at the bottom.
4. Add 40 µL of the Ligation Master Mix to each sample.

> Add the Ligation Master Mix last to reduce adapter dimers formation.

5. Mix by pipetting or vortexing, and briefly spin down the tubes to collect the liquid at the bottom.
6. Incubate the ligation reaction overnight (16 to 18 hours) on a thermocycler using the following program:

Temperature	Duration
16°C	∞

> Set the thermocycler lid to 50°C and volume to 110 µL (100 µL volume is acceptable if limited by thermocycler parameter).

Cleaning up post-ligation sample

To clean up post-ligation sample

Cleanup Beads V2 must be stored at the correct storage temperature to prevent degradation. Bead degradation may lead to incomplete DNA-to-bead binding during purification steps of the workflow, causing suboptimal assay performance, insufficient DNA yield, and loss of sample.

Cleanup Beads V2 must be equilibrated to room temperature and fully resuspended prior to use. If not, the probability for obtaining the correct bead-to-DNA ratio is low, which may result in capturing the incorrect size of DNA fragments, causing suboptimal assay performance, insufficient DNA yield, and loss of sample.

Cleanup Beads V2 are light-sensitive and should be protected from light when not in use. Excess light exposure may degrade its buffer, causing suboptimal assay performance, insufficient DNA yield, and loss of sample.

Prepare 80% ethanol fresh on the day of usage using 100% ethanol and nuclease-free water, PCR grade.

1. Remove the Cleanup Beads V2 from cold storage at least 15 minutes before use to bring them to room temperature.
2. Thoroughly mix the Cleanup Beads V2 by vortexing.
3. Add 88 µL of Cleanup Beads V2 to each sample.
4. Mix thoroughly by pipetting or vortexing, and quickly spin down the liquid without pelleting the beads.
5. Incubate for 10 minutes at room temperature.
6. Pellet beads on a 0.2 mL magnetic rack for 5 minutes. Visually inspect to ensure that all of the beads are collected to the side of the tube.
7. Discard the supernatant.
8. With beads still on the magnetic rack, wash the beads with 200 µL of freshly prepared 80% ethanol.
9. Incubate for at least 30 seconds at room temperature, and discard the ethanol.
10. Repeat *step 8* and *step 9* for a second wash with the freshly prepared 80% ethanol.
11. Spin down the tubes quickly to bring residual ethanol to the bottom without pelleting the beads.
12. Place the tubes on the magnetic rack to collect the beads. Remove residual ethanol using a P20 pipette.
13. Leave the tubes open on the magnetic rack to air dry the beads for 3 minutes.

Do not over dry the beads (visible by the formation of cracks on the pellet). Over drying the beads may lead to reduction in yield. Each bead pellet surface should have an uncracked matte appearance before resuspension.

14. Remove the tubes from the magnetic rack.
15. Add 20 µL nuclease-free water, PCR grade, to each sample on the beads, and resuspend by vortexing and quickly spinning down the sample.
16. Incubate for 2 minutes at room temperature.
17. Pellet beads on the magnetic rack for 3 minutes, and visually inspect to ensure that all of the beads are collected to the side of the tube.

18. Transfer the eluate into a new 0.2 mL strip tube. The eluate contains the adapter-ligated DNA sample.

Performing PCR amplification

To perform PCR amplification

1. Prepare the PCR Master Mix:

PCR Master Mix	
Reagent	**Volume per reaction**
PCR Reaction Mix (2X)	25 µL
PCR Primer Mix (10X)	5 µL

PCR Reaction Mix (2X) contains tetramethylammonium chloride. Follow safe laboratory practices when handling it.

2. Pipette 30 µL of the PCR Master Mix to the 20 µL of adapter-ligated sample.
3. Mix by vortexing, and briefly spin down the tubes to collect the liquid at the bottom.
4. Perform PCR on a thermocycler using the following cycling profile:

Thermocycler profile			
Stage	**Temperature**	**Duration**	**Cycles**
Initial denaturation	98°C	45 seconds	1
Denaturation	98°C	15 seconds	12
Annealing	60°C	30 seconds	
Extension	72°C	30 seconds	
Final extension	72°C	1 minute	1
Hold	4°C	∞	1

Set the thermocycler lid to 105°C and volume to 50 µL.

5. Proceed to *Cleaning up post-PCR product.*

Cleaning up post-PCR product

To clean up post-PCR product

Cleanup Beads V2 must be stored at the correct storage temperature to prevent degradation. Bead degradation may lead to incomplete DNA-to-bead binding during purification steps of the workflow, causing suboptimal assay performance, insufficient DNA yield, and loss of sample.

Cleanup Beads V2 must be equilibrated to room temperature and fully resuspended prior to use. If not, the probability for obtaining the correct bead-to-DNA ratio is low, which may result in capturing the incorrect size of DNA fragments, causing suboptimal assay performance, insufficient DNA yield, and loss of sample.

Cleanup Beads V2 are light-sensitive and should be protected from light when not in use. Excess light exposure may degrade its buffer, causing suboptimal assay performance, insufficient DNA yield, and loss of sample.

Prepare 80% ethanol fresh on the day of usage using 100% ethanol and nuclease-free water, PCR grade.

1. Remove the Cleanup Beads V2 from cold storage at least 15 minutes before use to bring them to room temperature.
2. Thoroughly mix the Cleanup Beads V2 by vortexing.

3. Add 50 µL of Cleanup Beads V2 to the PCR product.
4. Mix thoroughly by pipetting or vortexing, and quickly spin down the liquid without pelleting the beads.
5. Incubate for 10 minutes at room temperature.
6. Pellet beads on a 0.2 mL magnetic rack for 3 minutes, and visually inspect to ensure that all of the beads are collected to the side of the tube.
7. Discard the supernatant.
8. Remove the tubes from the magnetic rack, and resuspend the beads in 50 µL of nuclease-free water, PCR grade.
9. Rebind the DNA by adding an additional 50 µL of Cleanup Beads V2 to the 50 µL of sample from *step 8* to enhance cleanup of the ligated cfDNA sample.
10. Mix thoroughly by pipetting or vortexing, and quickly spin down the liquid without pelleting the beads.
11. Incubate for 10 minutes at room temperature.
12. Pellet beads on the magnetic rack for 3 minutes, and visually inspect to ensure all of the beads are collected to the side of the tube.
13. Discard the supernatant.
14. With beads still on the magnetic rack, wash the beads with 200 µL of freshly prepared 80% ethanol.
15. Incubate for at least 30 seconds at room temperature, and discard the ethanol.
16. Repeat *step 14* and *step 15* for a second wash with the freshly prepared 80% ethanol.
17. Spin down the tubes quickly to bring residual ethanol to the bottom without pelleting the beads.
18. Place the tubes on the magnetic rack to collect the beads. Remove residual ethanol using a P20 pipette.
19. Leave the tubes open on the magnetic rack to air dry the beads for 3 minutes.

Do not over dry the beads (visible by the formation of cracks on the pellet). Over drying the beads may lead to reduction in yield. Each bead pellet surface should have an uncracked matte appearance before resuspension.

19. Remove the tubes from the magnetic rack.
20. Add 65 µL nuclease-free water, PCR grade, to each sample, and resuspend thoroughly by pipetting or vortexing.
21. Incubate for 2 minutes at room temperature.
22. Pellet beads on the magnetic rack for 3 minutes, and visually inspect to ensure that all of the beads are collected to the side of the tube.
23. Transfer the eluate into a new 0.2 mL strip tube. The eluate contains the pre-capture library.

Safe stopping point: Freeze at -20°C for up to 1 month, or proceed to (*Suggested) Assessing library quality before enrichment*.

(Suggested) Assessing library quality before enrichment

The library preparation and enrichment procedures have been optimized for 10 to 50 ng of cfDNA input ranges. Library quantification and QC is not required to continue on to the enrichment protocol but may be useful for troubleshooting purposes. The volume unused for the enrichment procedure may be saved and used for library QC if troubleshooting is required.

(Suggested) To assess library quality before enrichment

1. Quantify the library yield by measuring 1 µL with the Qubit dsDNA HS Assay Kit according to the manufacturer's instructions.
2. Dilute 1 µL of the samples with nuclease-free water, PCR grade, to a recommended concentration for analysis using an Agilent High Sensitivity DNA Kit. A 10-fold dilution is generally sufficient.
3. Assess the average library size using an Agilent High Sensitivity DNA Assay on a 2100 Bioanalyzer following the manufacturer's instructions. The sample should show a primary peak at approximately 300 bp. See *Appendix B* for details.

Alternatively, an Agilent High Sensitivity D1000 ScreenTape assay on a 4200 TapeStation or a LabChip NGS 3K reagent kit with HT DNA X-Mark Chip on a LabChip GX Touch HT can be used.

The enrichment protocol has been optimized to tolerate moderate levels of adapter dimers. Residual adapter dimers are excluded from the library during the enrichment procedure.

Chapter 4. Performing the enrichment protocol

This section of the protocol uses the AVENIO ctDNA Enrichment Kit V2, the AVENIO Post-Hybridization Kit V2, and one or more of the AVENIO ctDNA Panels V2 (AVENIO ctDNA Targeted, Expanded, or Surveillance Panel V2). The purpose of this section is to enrich genes of interest from the ligated cfDNA samples produced in *Chapter 3*. Panels contain probes designed to target regions of interest for detection of cancer mutations present within ctDNA.

It is crucial to use the correct amount of V2 panel for the hybridization. Not enough panel being added to the hybridization reactions can lead to low on-target and/or reduced coverage post sequencing, and hence suboptimal assay performance and delayed results. Adding too much panel leads to little to no significant impact on sequencing and results.

It is important to ensure that the correct V2 panels are used for the new workflow conditions. The previous versions of the panels are not designed to hybridize properly using the new workflow conditions, and will lead to poor libraries and poor sequencing results, suboptimal assay performance, delayed results and potential loss of sample.

It is essential to use the correct V2 reagents (check for appropriate kit name and material/ lot number) because the new workflow is designed to provide optimal results with the REACH compliant reagents. The use of the previous version of reagents will lead to suboptimal assay performance, delayed results and potential loss of sample, resulting in poor libraries and poor sequencing results.

It is crucial to verify the shelf-life by checking the expiry date of reagents prior to use. Expired reagents/panel used in the workflow can lead to suboptimal assay performance, delayed results, potential loss of sample and the creation of poor libraries and poor sequencing results.

It is essential to use the correct hybridization temperature which was optimized when using the new Hybridization Buffer 2 V2 and V2 panels. The use of an incorrect hybridization temperature will affect target enrichment, which may result in suboptimal assay performance, delayed result, potential loss of sample, as well as poor sequencing results.

Change gloves when opening and closing strip tubes to minimize cross-contamination. Clearly label tubes at the required steps to minimize sample mix-up. Always check the reagent for correct name and expiration date before use.

For all master mixes, prepare 10% overage.

Preparing for hybridization

To prepare for hybridization

Cleanup Beads V2 must be stored at the correct storage temperature to prevent degradation. Bead degradation may lead to incomplete DNA-to-bead binding during purification steps of the workflow, causing suboptimal assay performance, insufficient DNA yield, and loss of sample.

Cleanup Beads V2 must be equilibrated to room temperature and fully resuspended prior to use. If not, the probability for obtaining the correct bead-to-DNA ratio is low, which may result in capturing the incorrect size of DNA fragments, causing suboptimal assay performance, insufficient DNA yield, and loss of sample.

Cleanup Beads V2 are light-sensitive and should be protected from light when not in use. Excess light exposure may degrade its buffer, causing suboptimal assay performance, insufficient DNA yield, and loss of sample.

80% ethanol should be prepared fresh on the day of usage using 100% ethanol and nuclease-free water, PCR grade.

1. Remove the Cleanup Beads V2 from cold storage at least 15 minutes before use to bring them to room temperature.
2. Transfer 30 µL of the pre-enrichment library from step 23 of the procedure *To clean up post-PCR product* to a new 0.2 mL strip tube to use for hybridization.

Freeze the remainder of the pre-enrichment library at -20°C for up to 1 month, or proceed to *Chapter 4, Performing the enrichment protocol*. This remainder can be used to repeat the enrichment if desired but will require additional hybridization reagents.

3. Add 20 µL of Hybridization Supplement to each sample. Mix by pipetting or vortexing, and briefly spin down the tubes to settle the liquid to the bottom.
4. Thoroughly mix the Cleanup Beads V2 by vortexing.
5. Add 100 µL of room temperature Cleanup Beads V2.
6. Mix thoroughly by pipetting or vortexing, and briefly spin down the liquid without pelleting the beads.
7. Incubate for 10 minutes at room temperature.
8. While incubating, prepare the Hybridization Master Mix:

Hybridization Master Mix	
Reagent	**Volume per reaction**
Hybridization Buffer 1 (2X)	31.1 µL
Hybridization Buffer 2 V2	10.9 µL
Nuclease-free water, PCR grade	1 µL

Hybridization Buffer 1 (2X) contains tetramethylammonium chloride. Follow safe laboratory practices when handling it.

9. Pellet beads on a 0.2 mL magnetic rack for 3 minutes, and visually inspect to ensure that all of the beads are collected to the side of the tube.
10. Discard the supernatant.
11. With the beads still on the magnetic rack, wash the beads with 200 µL of freshly prepared 80% ethanol.
12. Incubate for at least 30 seconds at room temperature, and discard the ethanol.
13. Spin down the tubes quickly to bring residual ethanol to the bottom without pelleting the beads.
14. Place the tubes on the magnetic rack to collect the beads. Remove residual ethanol using a P20 pipette.
15. Leave the tubes open on the magnetic rack to air dry the beads for 3 minutes.

Do not over dry the beads (visible by the formation of cracks on the pellet). Over drying the beads may lead to reduction in yield. Each pellet surface should have an uncracked matte appearance before resuspension.

16. Remove the tubes from the magnetic rack.
17. Add 13.4 µL of appropriate Enhancing Oligo to each sample, and resuspend beads by pipetting.

Enhancing Oligo number (EO#) must match the corresponding Sample Adapter number (SA#) used in the ligation step.

Do not multiplex samples during the hybridization. This will reduce assay performance.

18. After the beads are resuspended, add 43 µL of the Hybridization Master Mix.
19. Mix thoroughly by pipetting or vortexing, and quickly spin down the liquid without pelleting the beads.
20. Incubate for 2 minutes at room temperature.
21. Pellet beads on the magnetic rack for 3 minutes, and visually inspect to ensure that all of the beads are collected to the side of the tube.

Dispersed pelleting of the beads is frequently observed. Gently aspirate the supernatant to promote collecting as many beads by the magnet.

22. Transfer the supernatant to a new 0.2 mL strip tubes, and add 4 µL of the appropriate V2 Panel to each sample.

Check the label to ensure the correct panel is used: AVENIO ctDNA Targeted Panel V2, AVENIO ctDNA Expanded Panel V2, or AVENIO ctDNA Surveillance Panel V2. The correct panel name must be noted and entered in the AVENIO analysis software to properly analyze the data after sequencing. Each sample may receive a different panel, but only one panel is to be used per sample.

23. Vortex to mix the samples, and quickly spin down the tubes to settle the liquid to the bottom.
24. Incubate on a thermocycler overnight for 16 to 18 hours using the following hybridization program:

Hybridization program	
Temperature	**Duration**
95°C	10 minutes
60°C	∞

Set the thermocycler lid to 105°C and volume to 60 µL.

Preparing the Hybridization Wash Buffers

To prepare the Hybridization Wash Buffers

Hybridization Wash Buffers can be prepared immediately before performing the hybridization washes or up to 2 weeks prior to use. The 1X working solutions can be stored at room temperature for up to 2 weeks.

After preparing 1X working solutions of Stringent Wash Buffer, Hybridization Wash 1, Hybridization Wash 2, and Hybridization Wash 3, the buffers should be aliquoted into 0.2 mL strip tubes for ease of use.

1. Before the end of the hybridization, thaw the Hybridization Wash Buffers.

Ensure that stock wash buffers do not contain precipitates or are cloudy. If necessary, thoroughly vortex and gently warm precipitated or cloudy buffers at 37°C until buffers are completely clear. The Stringent Wash Buffer and Hybridization Wash 1 will have more precipitates.

2. Prepare the wash buffers according to the following table. The table indicates volumes required for 1 reaction. Include overage and scale accordingly for more reactions.

Buffer	Volume of stock buffer per reaction	Volume of PCR grade water per reaction	Total volume of 1X buffer prepared per sample	Temperature
Bead Wash Buffer (2.5X)	200 µL	300 µL	500 µL	Room temperature
Hybridization Wash 1 (10X)	10 µL	90 µL	100 µL	60°C
	20 µL	180 µL	200 µL	Room temperature
Stringent Wash Buffer (10X)	20 µL	180 µL	200 µL	60°C
	20 µL	180 µL	200 µL	60°C
Hybridization Wash 2 (10X)	20 µL	180 µL	200 µL	Room temperature
Hybridization Wash 3 (10X)	20 µL	180 µL	200 µL	Room temperature

3. Place the strips of the 1X working solutions of the Stringent Wash Buffers and Hybridization Wash 1 specified at 60°C in the thermocycler so that they may incubate for at least 15 minutes while the streptavidin beads are being prepared.

Preparing the streptavidin beads

To prepare the streptavidin beads

1. Allow the Dynabeads M-270 Streptavidin Beads to reach room temperature for 30 minutes prior to use.
2. Vortex the streptavidin beads thoroughly for 15 seconds before use to ensure they are resuspended well.
3. In a new 1.5 mL microcentrifuge tube, aliquot 100 µL of streptavidin beads for each capture reaction (100 µL beads for 1 reaction; 400 µL beads for 4 reactions).

> Prepare up to 4 reactions in each 1.5 mL microcentrifuge tube. 16 reactions will require 4 tubes.

4. Pellet the streptavidin beads on a 1.5 mL magnetic rack until the solution is clear, and discard the supernatant.
5. Add 200 µL of 1X Bead Wash Buffer per capture (200 µL for 1 reaction; 800 µL for 4 reactions).
6. Thoroughly wash streptavidin beads by vortexing 2 times for 10 seconds each, and quickly spin down the liquid without pelleting the beads.

> **Dynabeads M-270 Streptavidin Beads must be washed thoroughly for optimal assay performance.**

7. Pellet the streptavidin beads on the magnetic rack until the solution is clear and discard the clear supernatant.
8. Repeat *step 5* through *step 7* for a total of two washes.
9. Remove the tubes from the magnetic rack, and resuspend the beads with 100 µL of 1X Bead Wash Buffer per capture (100 µL for 1 reaction; 400 µL for 4 reactions).
10. Combine all beads in a new 2.0 mL tube.
11. Mix thoroughly by vortexing 2 times for 5 seconds each, and aliquot 100 µL of resuspended beads per sample into new 0.2 mL strip tubes.

> Streptavidin beads settle quickly. To ensure even distribution and performance, vortex the tube of beads every 3 aliquots.

12. Pellet the streptavidin beads on a 0.2 mL magnetic rack until the solution is clear, and discard the supernatant.

13. Proceed immediately to Performing the hybridization cleanup.

Do not allow the beads to dry at this stage. Small amounts of residual 1X Bead Wash Buffer will not interfere with subsequent steps.

Performing the hybridization cleanup

To perform the hybridization cleanup

Use prepared 1X buffers for all wash steps.

1. Ensure that the following buffers are aliquoted into 0.2 mL strip tubes at the indicated volumes, and incubate the buffers specified at 60°C in the thermocycler for at least 15 minutes prior to use.

Buffer	Volume	Temperature
Hybridization Wash 1	100 µL	60°C
Stringent Wash Buffer	200 µL	60°C
Stringent Wash Buffer	200 µL	60°C
Hybridization Wash 1	200 µL	Room temperature
Hybridization Wash 2	200 µL	Room temperature
Hybridization Wash 3	200 µL	Room temperature

2. Remove the hybridization reaction from the thermocycler. Working quickly, briefly spin down the liquid to the bottom of the tube, and transfer the 60.4 µL hybridization reaction onto the pelleted streptavidin beads.

Work quickly to minimize cooling of the hybridization reaction, which may reduce assay performance. If processing multiple strips, it is recommended to process 1 to 2 strips at a time, while keeping the rest of the strips on the thermocycler at 60°C.

3. Mix thoroughly by vortexing, and quickly spin down the liquid without pelleting the streptavidin beads.
4. Place the samples back on the thermocycler, and incubate at 60°C for 15 minutes to bind the hybridized DNA to the streptavidin beads.
5. Remove the strip of samples and the pre-heated Hybridization Wash 1 from the thermocycler.
6. Quickly transfer 100 µL of pre-heated Hybridization Wash 1 to the sample, and immediately mix by pipetting thoroughly up and down 20 times. **Do not vortex the samples.**

When pipetting up and down, avoid bubbles, which can reduce the washing efficiency. If necessary, adjust the pipetting volume to reduce bubbles.

7. Quickly spin down the liquid, and place the samples on the 0.2 mL magnetic rack to pellet the beads for 1 minute. When the supernatant is clear, discard the supernatant.
8. Quickly remove the pre-heated Stringent Wash Buffer from the thermocycler, and remove the strip of samples from the magnetic rack.
9. Immediately transfer 200 µL of pre-heated Stringent Wash Buffer to the sample. Mix thoroughly by pipetting up and down 20 times or by vortexing for 5 seconds. Quickly spin down the liquid without pelleting the beads.
10. Incubate the samples at 60°C for 5 minutes on the thermocycler.
11. Remove the strip of samples from the thermocycler and quickly spin down the liquid. Place the tubes on the magnetic rack to pellet the beads for 1 minute. When the supernatant is clear, discard the supernatant.
12. Repeat *step 8* through *step 11* for a second Stringent Wash.
13. Remove the strip of samples from the magnetic rack, and add 200 µL of room temperature Hybridization Wash 1.
14. Mix the pellet thoroughly by pipetting up and down 20 times. **Do not vortex the samples.**
15. Quickly spin down the liquid without pelleting the beads and incubate for 1 minute.
16. Place the tubes on the magnetic rack to pellet the beads for 1 minute. When the supernatant is clear, discard the supernatant.

17. Remove the strip of samples from the magnetic rack.

18. Add 200 µL of room temperature Hybridization Wash 2 to each sample.

19. Mix the pellet thoroughly by pipetting 20 times or by vortexing for 5 seconds, and quickly spin down the liquid without pelleting the beads.

20. Carefully transfer all of the contents to a new 0.2 mL strip tube. Incubate for 1 minute at room temperature.

Transferring to a fresh tube helps to ensure thorough washing. Ensure that all of the beads are transferred and no pellet is left behind.

21. Place the tubes on the magnetic rack to pellet the beads for 1 minute. When the supernatant is clear, discard the supernatant.

22. Remove the strip of samples from the magnetic rack and add 200 µL of room temperature Hybridization Wash 3 to each sample.

23. Mix the pellet thoroughly by pipetting up and down 20 times or by vortexing for 5 seconds.

24. Quickly spin down the liquid without pelleting the beads, and incubate for 1 minute at room temperature.

25. Place the tubes on the magnetic rack to pellet the beads for 1 minute. When the supernatant is clear, discard the supernatant.

Remove as much of the Hybridization Wash 3 as possible to avoid PCR inhibition.

26. Remove the tubes from the magnetic rack, and resuspend the streptavidin beads by adding 20 µL of nuclease-free water, PCR grade, to each sample, and incubate for 2 minutes.

Do not discard streptavidin beads at this point. Enriched DNA remains bound to the streptavidin beads.

27. Proceed to *Amplifying the enriched ctDNA sample*.

Amplifying the enriched ctDNA sample

To amplify the enriched ctDNA sample

1. Prepare the PCR Master Mix:

PCR Master Mix	
Reagent	**Volume per reaction**
PCR Reaction Mix (2X)	25 µL
PCR Primer Mix (10X)	5 µL

PCR Reaction Mix (2X) contains tetramethylammonium chloride. Follow safe laboratory practices when handling it.

2. Add 30 µL of the PCR Master Mix to the 20 µL of DNA-bound streptavidin beads sample.

3. Briefly vortex and spin down the tubes to bring the liquid to the bottom.

4. Transfer the entire reaction to a new 0.2 mL strip tube.

5. Perform PCR on a thermocycler using the following cycling profile:

	Thermocycler profile		
Stage	**Temperature**	**Duration**	**Cycles**
Initial denaturation	98°C	45 seconds	1
Denaturation	98°C	15 seconds	15
Annealing	60°C	30 seconds	
Extension	72°C	30 seconds	
Final extension	72°C	1 minute	1
Hold	4°C	∞	1

Set the thermocycler lid to 105°C and volume to 50 µL.

6. After PCR, proceed to *Cleaning up post-capture PCR product*.

Cleaning up post-capture PCR product

To clean up post-capture PCR

Cleanup Beads V2 must be stored at the correct storage temperature to prevent degradation. Bead degradation may lead to incomplete DNA-to-bead binding during purification steps of the workflow, causing suboptimal assay performance, insufficient DNA yield, and loss of sample.

Cleanup Beads V2 must be equilibrated to room temperature and fully resuspended prior to use. If not, the possibility for obtaining the correct bead-to-DNA ratio is low, which may result in capturing the incorrect size of DNA fragments, causing suboptimal assay performance, insufficient DNA yield, and loss of sample.

Cleanup Beads V2 are light-sensitive and should be protected from light when not in use. Excess light exposure may degrade its buffer and lead to loss of sample, causing suboptimal assay performance, insufficient DNA yield, and loss of sample.

Prepare 80% ethanol fresh on the day of usage using 100% ethanol and nuclease-free water, PCR grade.

1. Remove the Cleanup Beads V2 from cold storage at least 15 minutes before use to bring them to room temperature.
2. Resuspend amplified sample containing the streptavidin beads by vortexing, and quickly spin down the liquid without pelleting the beads.
3. Place the amplified samples containing the streptavidin beads on the magnetic rack for 3 minutes until the supernatant is clear.
4. Carefully transfer the supernatant, which contains the PCR amplified, panel-enriched library, to a new 0.2 mL strip tube. Discard the streptavidin beads.

Pay attention to not discard the supernatant, which contains the PCR amplified, panel-enriched library.

A cloudy supernatant may be observed; however, this occurrence does not impact downstream yield or performance.

5. Thoroughly mix the Cleanup Beads V2 by vortexing.
6. Add 50 µL of Cleanup Beads V2 to the PCR product.
7. Mix thoroughly by pipetting or vortexing, and quickly spin down the liquid without pelleting the beads.
8. Incubate for 10 minutes at room temperature.
9. Pellet beads on the magnetic rack for 3 minutes, and visually inspect to ensure that all of the beads are collected to the side of the tube.
10. Discard the supernatant.
11. With beads still on the magnetic rack, wash the beads with 200 µL of freshly prepared 80% ethanol.

12. Incubate for at least 30 seconds at room temperature and discard the ethanol.
13. Repeat *step 11* and *step 12* for a second wash.
14. Spin down the tubes quickly to bring residual ethanol to the bottom without pelleting the beads.
15. Place the tubes on the magnetic rack to collect the beads. Remove residual ethanol using a P20 pipette.
16. Leave the tubes open on the magnetic rack to air dry the beads for 3 minutes.

Do not over dry the beads (visible by the formation of cracks on the pellet). Over drying the beads may lead to reduction in yield. Each bead pellet surface should have an uncracked matte appearance before resuspension.

17. Remove the tubes from the magnetic rack, and add 50 µL nuclease-free water, PCR grade, to each sample and resuspend thoroughly by pipetting up and down.
18. Incubate for 2 minutes at room temperature.
19. Pellet the beads on the magnetic rack for 3 minutes, and visually inspect to ensure that all of the beads are collected to the side of the tube.
20. Transfer the clear eluate into a new 0.2 mL strip tube. The eluate contains the final library, ready for QC.

Freeze at –20°C for up to 1 month, or proceed to *Assessing enriched library quality*.

Assessing enriched library quality

To assess enriched library quality

1. Quantify the concentration of the individual enriched libraries with the Qubit dsDNA HS Assay Kit according to the manufacturer's instructions.

The typical concentration of the enriched library is between 0.5 ng/µL and 30 ng/µL, given 50 ng input. If the concentration is outside of this range, see *Appendix C*. Less cfDNA input will yield less concentration of enriched library. 10 ng of cfDNA input typically yields library concentrations of 0.3 to 20 ng/µL.

2. If needed, dilute 1 µL of the enriched libraries with nuclease-free water, PCR grade, to bring the concentration within the Bioanalyzer High Sensitivity specifications. A 5 to 10-fold dilution is generally sufficient.
3. Assess the average library size using an Agilent High Sensitivity DNA Assay on a 2100 Bioanalyzer following the manufacturer's instructions.

Alternatively, a High Sensitivity D1000 ScreenTape assay on a 4200 TapeStation or a LabChip NGS 3K reagent kit with HT DNA X-Mark Chip on a LabChip GX Touch HT can be used.

The ideal peak size of the library is ~300 bp. There should be minimal to no adapter dimers present (see *Appendix B*).

4. Freeze at –20°C for up to 1 month, or proceed to *Chapter 5, DNA sequencing*.

Chapter 5. DNA sequencing

The AVENIO ctDNA Analysis Kits V2 prepare sequencing-ready libraries from cfDNA isolated from plasma. After QC of the enriched libraries from *Chapter 4*, the samples are ready for pooling and sequencing with the Illumina NextSeq 500/550 instrument or NextSeq 550Dx instrument in RUO Mode.

The Illumina NextSeq 500/550/550Dx instrument is a third-party instrument. This guide makes no claims on the instrument, and it is not supported by Roche Customer Support.

Ensure that the following is available:

- The Illumina NextSeq 500/550/500Dx instrument is configured in standalone mode
- Illumina Sequencing Kit listed under *Consumables purchased from other vendors*
    - NextSeq 500 500/550 High Output Kit v2.5 (300 cycles)
    - NextSeq 500Dx High Output Kit v2.5 (300 cycles)
    - PhiX Sequencing Control V3
- PCR grade water
- 1 M NaOH, molecular biology grade
- 200 mM Tris-HCl, pH 7.0, molecular biology grade

Pooling enriched DNA samples for sequencing

Ensure that each sample was prepared with a unique Sample Adapter #.

Up to 16 samples can be pooled and sequenced as 1 multiplexed pool per NextSeq 500/550/550Dx sequencing run.

Although 16 samples can be multiplexed to achieve high sensitivity, assay performance may be further enhanced by multiplexing fewer samples to reach greater sequencing depth.

Avoid pooling biological cfDNA samples with cell line or engineered DNA samples in the same run. Cell line or engineered DNA may contain poorly characterized variants and may harbor abnormally high allele fraction mutations, which may interfere with the sequencing of biological cfDNA samples.

Avoid pooling AVENIO Tumor Tissue libraries, AVENIO ctDNA libraries or non-AVENIO libraries in the same NextSeq run. Mixing libraries generated from different workflows will make the final sequencing data unanalyzable by the AVENIO Oncology Analysis Software.

To pool enriched DNA samples for sequencing

1. Use the concentration and the average library size determined in *Assessing enriched library quality* to calculate the molarity of each sample in nM using the following equation:

   Library Molarity in nM = (library concentration in ng/µL) / ((average library size in bp*607.4)+157.9)*10^6

   For example, if library concentration = 10 ng/µL and average library size is 300 bp, then the Library Molarity in nM = 10/((300*607.4)+157.9)*10^6= 54.83.

2. Pool an appropriate volume of each sample so that there is an equal molar mass of each sample in the pool. Prepare enough total volume sufficient for Illumina's suggested denaturing protocol (for an example, refer to *Illumina's NextSeq System Denature and Dilute Libraries Guide)*.

   It is important to pool equal molar mass per sample, to ensure that each sample receives approximately an equal amount of sequencing reads for data analysis.

   Ensure that each sample to be pooled for sequencing used a Sample Primer ID unique to the pool. Mixing more than one sample with the same Sample Primer ID will make final sequencing data for these samples unusable.

3. Vortex and spin-down pooled libraries briefly.
4. Quantify the concentration of the pooled libraries using the Qubit dsDNA HS Assay Kit according to the manufacturer's instructions.
5. Assess the average fragment size of the pooled libraries by diluting to a recommended concentration for analysis using Agilent High Sensitivity DNA Kit on a 2100 Bioanalyzer following the manufacturer's instructions.

   Alternatively, a High Sensitivity D1000 ScreenTape assay on a 4200 TapeStation or a LabChip NGS 3K reagent kit with HT DNA X-Mark Chip on a LabChip GX Touch HT can be used.

6. Calculate the molarity of the pooled library outlined in *step 1.*
7. Dilute the pooled library following Illumina's NextSeq guideline.
8. Proceed to *Denaturing enriched DNA pool and prepare for sequencing.*

Denaturing enriched DNA pool and prepare for sequencing

To denature enriched DNA pool and prepare for sequencing

1. Follow Illumina's suggested protocol to denature and dilute libraries for NextSeq sequencing. (For an example, refer to Illumina's *NextSeq System Denature and Dilute Libraries Guide*.)

2. Use a 15% PhiX spike-in sequencing control. Prepare PhiX control library, and spike in 15% PhiX by volume as described in Illumina's *NextSeq System Denature and Dilute Libraries Guide*.

   It is the user's responsibility to determine the optimal loading amount onto the NextSeq 500/550/550Dx instrument to attain Illumina's recommended cluster density.

   After sequencing, aligned PhiX levels of 5 to 15% will yield quality results.

Sequencing multiplexed libraries with the Illumina NextSeq 500/550/550Dx

To sequence multiplexed libraries with the Illumina NextSeq 500/550/550Dx

1. Use Illumina's *NextSeq 500/550 System Guide* or *NextSeq 550Dx Instrument Guide* for loading instructions for paired-end 150-cycle run sequencing.

The steps on the Run Setup screen differs based on the system configuration. The NextSeq 500/550 instrument must be configured to Manual Mode. If using the NextSeq 550Dx, use the instrument in the Research Mode. Use of Basespace mode is not supported.

2. Use the following run parameters:

- **Run name**: User defined
- **Library ID**: User defined
- **Recipe**: NextSeq High
- **Read 1**: 151 cycles
- **Read 2**: 151 cycles
- **Index 1**: 8 cycles

The AVENIO ctDNA Analysis Kit only supports sequencing of single indexed samples. Do not select or insert cycle number for Index 2.

The steps on the Run Setup screen differs based on the system configuration. The NextSeq 500/550 instrument must be configured to standalone mode. Use of Basespace mode is not supported.

Performing Illumina NextSeq 500/550/550Dx maintenance

To perform Illumina NextSeq 500/550/550Dx maintenance

1. Follow Illumina's manufacturer recommendations for regular instrumentation maintenance.
2. Frequently perform the Manual Post-Run Wash according to Illumina's protocols.

Regular washing of the Illumina NextSeq 500/550/550Dx reduces the possibility of contamination of samples from past sequencing runs.

Performing post-sequencing data analysis

To perform post-sequencing data analysis

Refer to the *AVENIO Oncology Analysis Software User Guide.*

Appendix A. AVENIO ctDNA Analysis Kits V2

The AVENIO ctDNA Analysis Kits V2 include the following products:

- *AVENIO ctDNA Targeted Kit V2*
- *AVENIO ctDNA Expanded Kit V2*
- *AVENIO ctDNA Surveillance Kit V2*

Each product contains a uniquely designed probe panel that target different regions in the genome.

Cleanup Beads V2 are shipped frozen but should be stored at 2°C to 8°C. They are located within the AVENIO ctDNA Library Prep Kit V2 and the AVENIO ctDNA Enrichment Kit V2. Upon shipment arrival, remove the vials from both kit boxes, and transfer the Cleanup Beads V2 to 2°C to 8°C for storage.

Cleanup Beads V2 are light-sensitive and should be protected from light when not in use. Excess light exposure may degrade its buffer and lead to loss of sample, causing suboptimal assay performance, insufficient DNA yield, and loss of sample.

AVENIO ctDNA Targeted Kit V2

The AVENIO ctDNA Targeted Kit V2 includes:

Orderable kit name	Box #	Sub-kit name	Shipping	Storage
AVENIO ctDNA Targeted Kit V2	1	AVENIO cfDNA Isolation Kit V2	15°C to 30°C	15°C to 30°C
	2	AVENIO ctDNA Library Prep Kit V2	-15°C to -25°C	-15°C to -25°C
	3	AVENIO ctDNA Enrichment Kit V2	-15°C to -25°C	-15°C to -25°C
	4	AVENIO ctDNA Targeted Panel V2	-15°C to -25°C	-15°C to -25°C
	5	AVENIO Post-Hybridization Kit V2	-15°C to -25°C	-15°C to -25°C

AVENIO ctDNA Expanded Kit V2

The AVENIO ctDNA Expanded Kit V2 includes:

Orderable kit name	Box #	Sub-kit name	Shipping	Storage
AVENIO ctDNA Expanded Kit V2	1	AVENIO cfDNA Isolation Kit V2	15°C to 30°C	15°C to 30°C
	2	AVENIO ctDNA Library Prep Kit V2	-15°C to -25°C	-15°C to -25°C
	3	AVENIO ctDNA Enrichment Kit V2	-15°C to -25°C	-15°C to -25°C
	4	AVENIO ctDNA Expanded Panel V2	-15°C to -25°C	-15°C to -25°C
	5	AVENIO Post-Hybridization Kit V2	-15°C to -25°C	-15°C to -25°C

AVENIO ctDNA Surveillance Kit V2

The AVENIO ctDNA Surveillance Kit V2 includes:

Orderable kit name	Box #	Sub-kit name	Shipping	Storage
AVENIO ctDNA Surveillance Kit V2	1	AVENIO cfDNA Isolation Kit V2	15°C to 30°C	15°C to 30°C
	2	AVENIO ctDNA Library Prep Kit V2	-15°C to -25°C	-15°C to -25°C
	3	AVENIO ctDNA Enrichment Kit V2	-15°C to -25°C	-15°C to -25°C
	4	AVENIO ctDNA Surveillance Panel V2	-15°C to -25°C	-15°C to -25°C
	5	AVENIO Post-Hybridization Kit V2	-15°C to -25°C	-15°C to -25°C

AVENIO ctDNA Analysis Kits V2 - Sub-Kits

The AVENIO ctDNA Targeted V2, Expanded V2, and Surveillance V2 Kit each contain the following sub-kits:

AVENIO cfDNA Isolation Kit V2

The AVENIO cfDNA Isolation Kit V2 supports up to 16 samples.

Component	Quantity
Proteinase K	100 mg
DNA PBB V2	10 mL
Wash Buffer I	25 mL
Wash Buffer II	12.5 mL
DNA Elution Buffer	6 mL
High Pure Extender Assembly Unit	20
Collection Tubes	75

AVENIO ctDNA Library Prep Kit V2

The AVENIO ctDNA Library Prep Kit V2 supports up to 16 samples.

Component	Quantity
DNA Preparation Buffer	210 μL
DNA Preparation Enzyme V2	100 μL
Ligation Buffer	900 μL
DNA Ligase	300 μL
PCR Reaction Mix (2X)	690 μL
PCR Primer Mix (10X)	138 μL
Sample Adapter 1 ~ Sample Adapter 16	15 μL
Cleanup Beads V2	6.8 mL

AVENIO ctDNA Enrichment Kit V2

The AVENIO ctDNA Enrichment Kit V2 supports up to 16 samples.

Component	Quantity
Hybridization Supplement	1000 μL
Hybridization Buffer 1 (2X)	890 μL
Hybridization Buffer 2 V2	500 μL
Enhancing Oligo 1 ~ Enhancing Oligo 16	20 μL
Cleanup Beads V2	6.8 mL

AVENIO ctDNA Targeted Panel V2

The AVENIO ctDNA Targeted Panel V2 supports up to 16 samples.

Component	Quantity
AVENIO ctDNA Targeted Panel V2	64 μL

AVENIO ctDNA Expanded Panel V2

The AVENIO ctDNA Expanded Panel V2 supports up to 16 samples.

Component	Quantity
AVENIO ctDNA Expanded Panel V2	64 μL

AVENIO ctDNA Surveillance Panel V2

The AVENIO ctDNA Surveillance Panel V2 supports up to 16 samples.

Component	Quantity
AVENIO ctDNA Surveillance Panel V2	64 μL

AVENIO Post-Hybridization Kit V2

The AVENIO Post-Hybridization Kit V2 supports up to 24 samples.

Component	Quantity
Bead Wash Buffer (2.5X)	12 mL
Hybridization Wash 1 (10X)	900 µL
Hybridization Wash 2 (10X)	580 µL
Hybridization Wash 3 (10X)	580 µL
Stringent Wash Buffer (10X)	1160 µL
PCR Reaction Mix (2X)	690 µL
PCR Primer Mix (10X)	138 µL

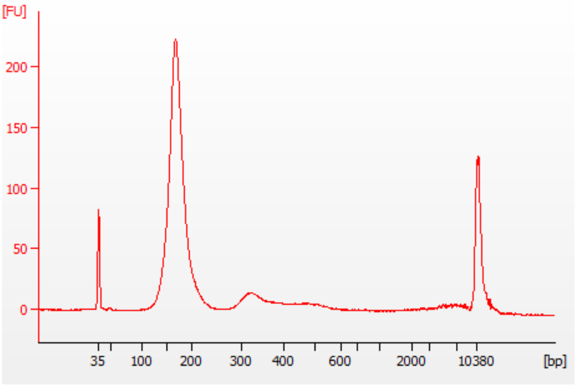
Appendix B. QC example profiles

This appendix shows examples of Bioanalyzer profiles for good and poor quality samples that have undergone QC, as indicated within the protocol. The profiles provided are from internal data at Roche. Similar profiles are observed on the TapeStation and LabChip.

QC of cfDNA isolated from plasma (Chapter 2)

Typical cfDNA Bioanalyzer profile

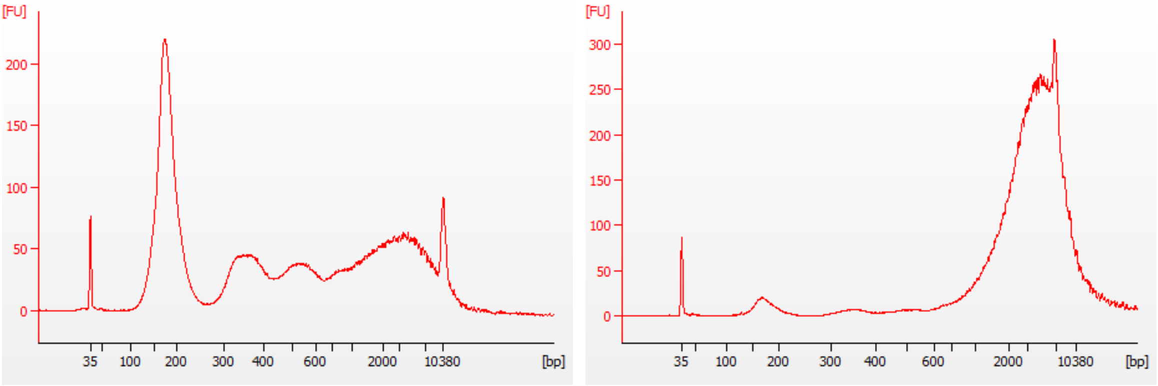
Example A



Bioanalyzer profiles for good quality cfDNA consist of a major peak at 160 to 200 bp. A smaller secondary peak for cfDNA may be visible at 320 to 400 bp, and a tertiary cfDNA peak may be visible at ~ 540 bp. Good quality cfDNA will consist of no or minimal high molecular weight genomic DNA (>2000 bp).

Typical poor quality cfDNA Bioanalyzer profile

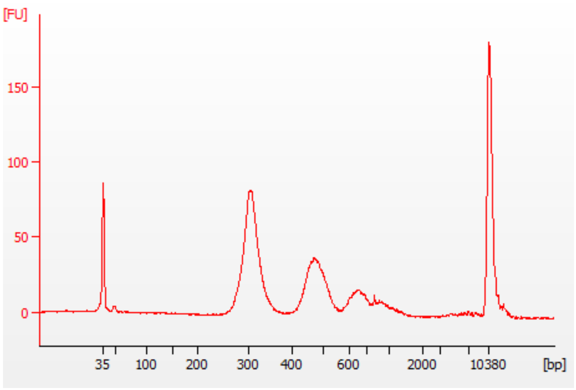
Example B **Example C**



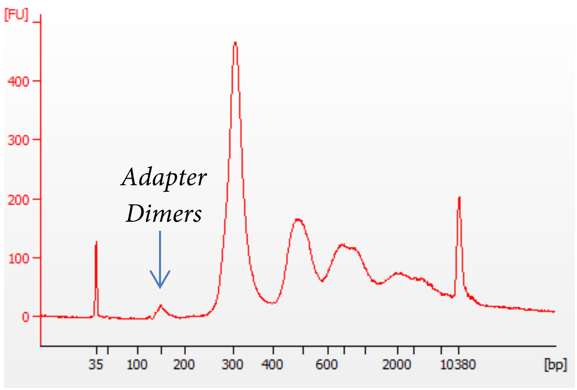
Poor quality cfDNA will show an accumulation of high molecular weight genomic DNA from 600 to >10,000 bp. In Example B, the genomic DNA may artificially increase the secondary and tertiary ctDNA peak. In Example C, the contaminating DNA is significantly greater than the primary ctDNA peak (~180 bp). Poor quality cfDNA can be due to poor quality plasma sample, sub-optimal blood and plasma collection techniques, sub-optimal blood and plasma storage, slow processing of plasma separation, or poor separation of plasma from leukocytes during plasma collection. It is not recommended to use DNA with these profiles as input into the library preparation procedure.

QC of pre-enrichment libraries (Chapter 3)

Typical 2100 Bioanalyzer profiles of a pre-enriched library

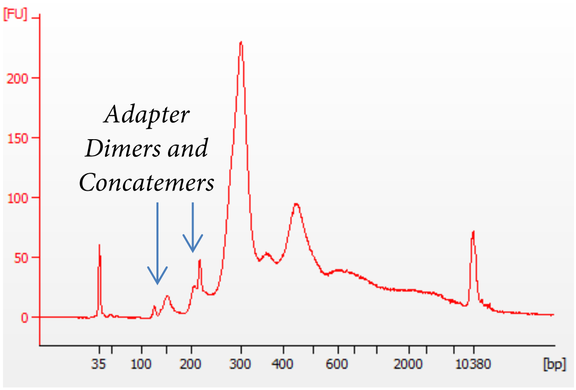


Example D



Example E

Example D and Example E show the primary ctDNA peak shifted to ~300 bp due to the increase in molecular weight after successful Sample Adapter ligation. In addition to the major peak at ~300 bp, several minor peaks will be enhanced and may be visible at >300 bp. Example E shows the presence of minimal adapter dimers near 150 bp.

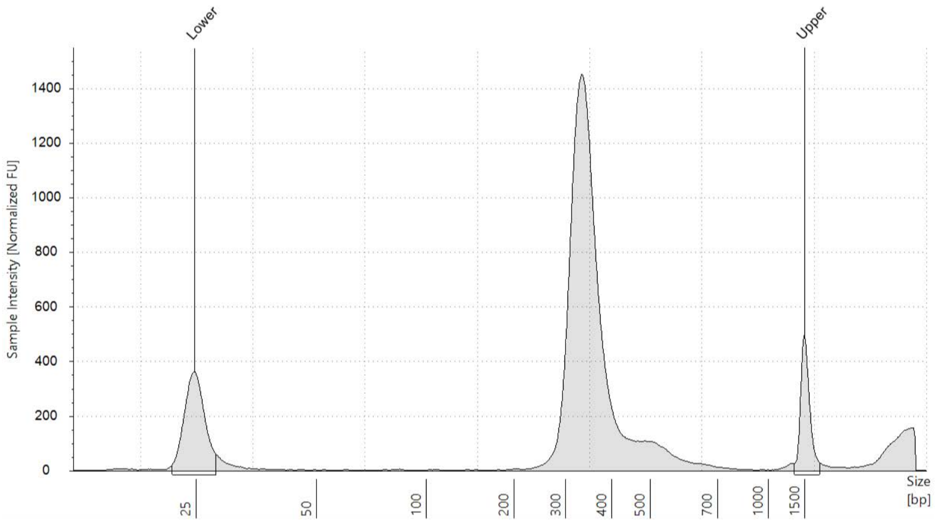


Example F

Example F is a Bioanalyzer trace of a pre-enriched library where there is an acceptable level of adapter dimers at ~150 bp and ~210 bp. The sample can move forward to the enrichment procedure. The assay is able to tolerate moderate levels of adapter dimers.

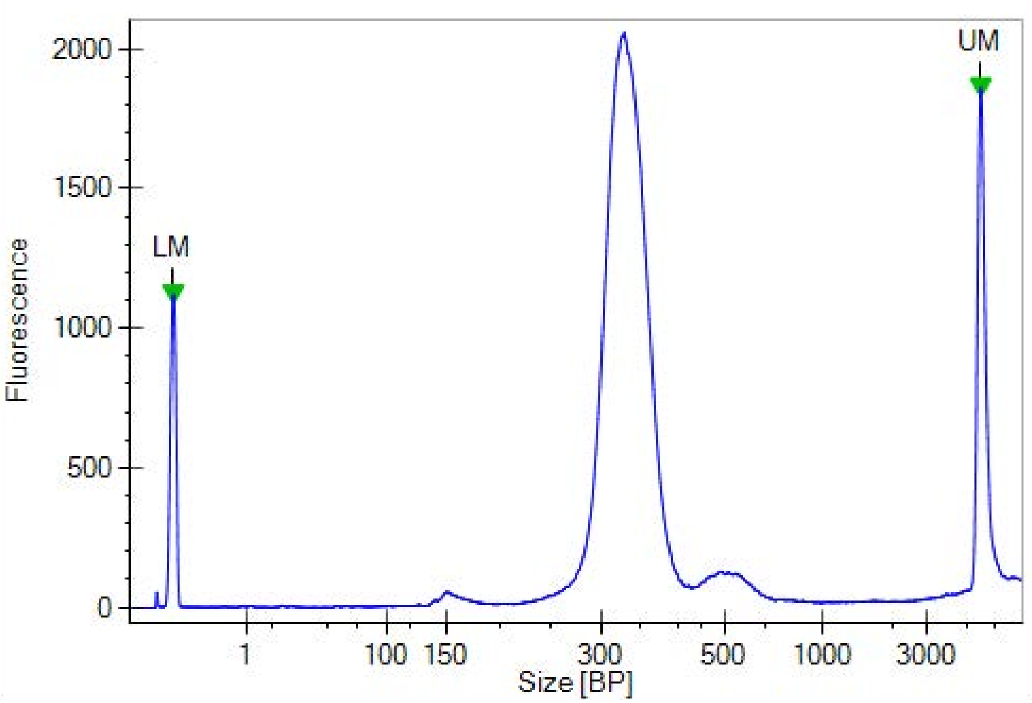
Typical TapeStation profiles of a pre-enriched library

The following is an example of a TapeStation profile showing a typical, good adapter-ligated library.



Typical LabChip profiles of a pre-enriched library

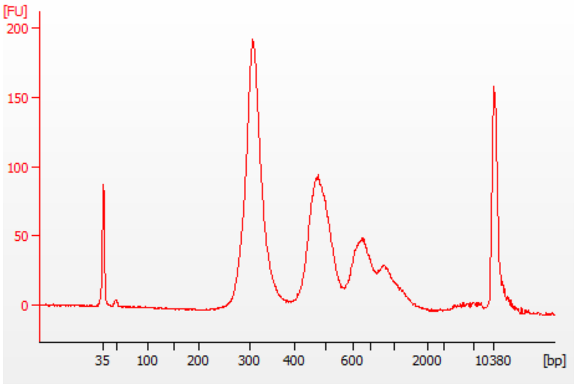
The following is an example of a LabChip profile showing a typical, good adapter-ligated library.



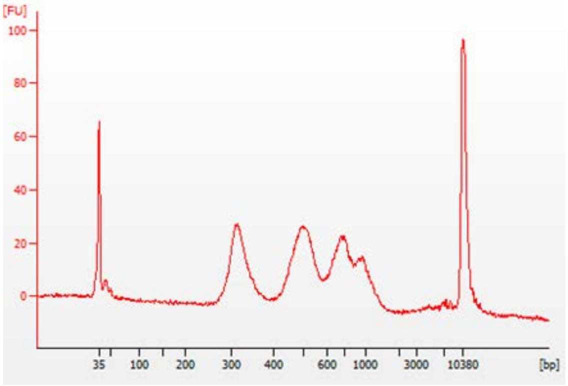
QC of final sequencing libraries (Chapter 4)

Typical Bioanalyzer profiles of final sequencing libraries

Example G



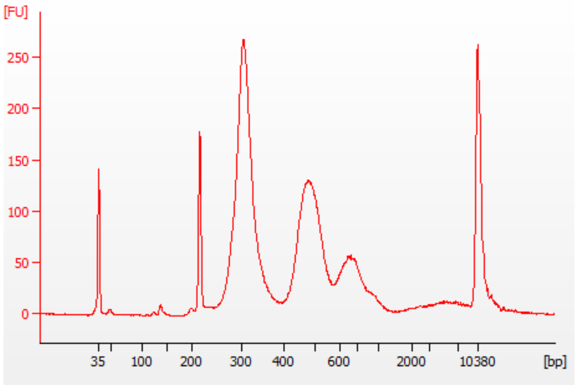
Example H



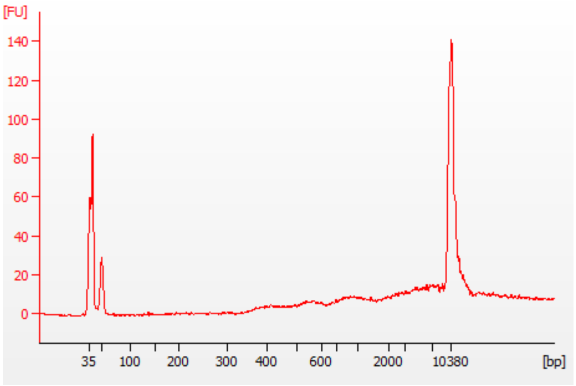
Example G shows a typical good library Bioanalyzer profile with no to negligible adapter dimers and distinguishable ctDNA peaks with a size shift to indicate successful adapter ligation. The ligated libraries show the primary ctDNA peak at ~300 bp, with additional larger ctDNA peaks. Example H is a Bioanalyzer profile of a sequencing library that is still suitable for sequencing. Although the 300 bp peak is lower, the sample is still free of adapter dimers. The 300 bp peak may be lower due to loss during the PCR cleanup step. This may result in a lower Unique Depth when sequenced, but the library will still sequence well on the NextSeq 500/550.

Typical poor Bioanalyzer profiles of final sequencing library

Example I



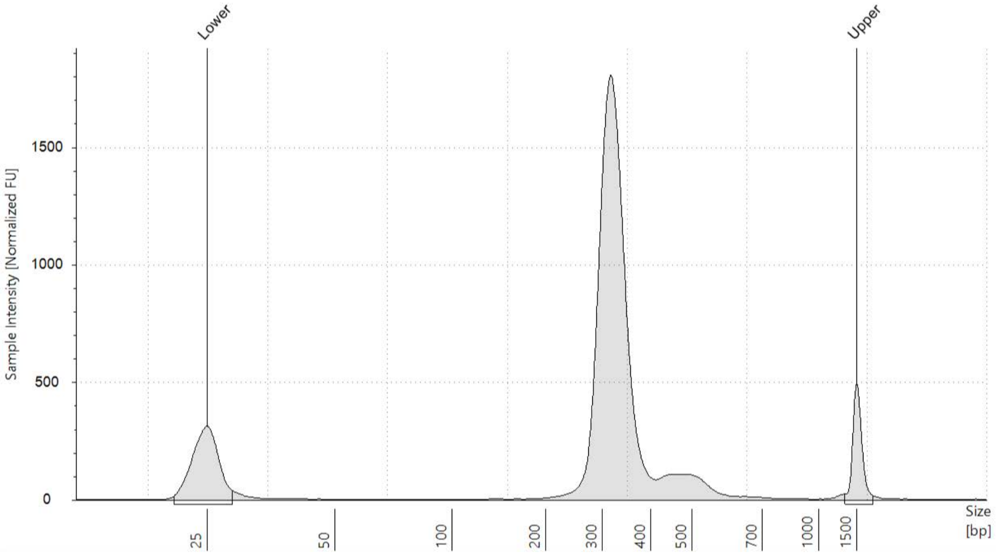
Example J



Example I is a poor library that has residual adapter dimers at a high level (~210 bp). This QC is indicative that the enrichment procedure may have failed. Note that extremely high levels of adapter dimers input into the sequencer may also fail sequencing runs – it is not recommended to sequence the sample with such high adapter dimers. Example J shows a sample with no post-capture ctDNA library. This could be due to multiple reasons: failure of captured library to bind to the streptavidin beads, no panel added, or incomplete washing. Ensure that the Bioanalyzer is working properly and the sample was properly diluted and added onto the Bioanalyzer chip.

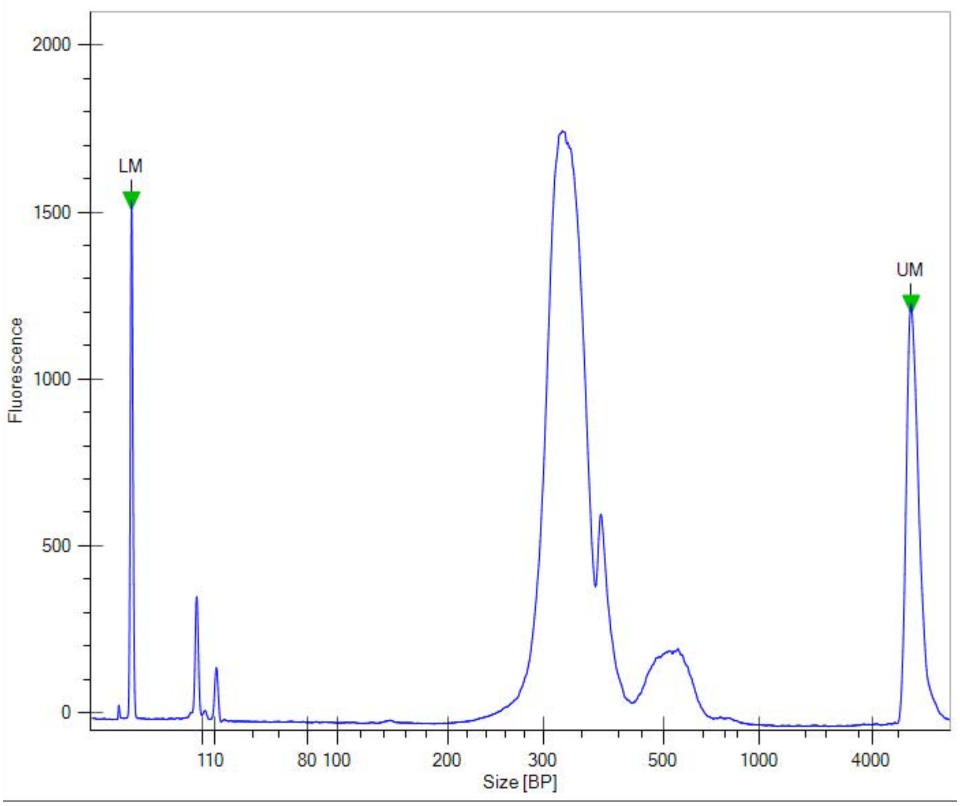
Typical TapeStation profiles of final sequencing libraries

The following is an example of a TapeStation profile showing a typical, good final enriched library.



Typical LabChip profiles of final sequencing libraries

The following is an example of a LabChip profile showing a typical, good final enriched library.



Appendix C. Troubleshooting

Pre-sequencing QC troubleshooting

Issue	Cause	Possible resolution
No or low yield from cfDNA isolation	Poor plasma source	■ Ensure proper handling and storage of blood and plasma samples. ■ It is possible that the sample naturally has low cfDNA content. Input more sample volume.
High molecular weight DNA present in isolated cfDNA	Poor plasma source	■ Ensure proper handling and storage of blood and plasma samples. Process the blood quickly after collection to minimize the amount of genomic DNA in the final eluate. ■ Ensure adequate centrifugation to effectively separate the plasma layer. ■ Avoid pipetting the buffy coat layer when collecting the plasma. ■ After thawing plasma, centrifuge down remaining cellular debris.
High adapter dimers in the pre-enrichment library, after the first PCR	Insufficient cfDNA used in the assay	■ Ensure proper quantification of the input cfDNA. ■ Add more cfDNA input into library preparation. ■ Ensure that there is minimal genomic DNA in the isolation eluate.
	Poor clean-up	■ Ensure proper volumes are used in the reaction. ■ Clean the sample again, maintaining the sample-to-bead ratio. Note that this additional cleanup may result in some sample loss and lower Unique Depth.
Abnormal Bioanalyzer profile of pre-enrichment library	High molecular weight DNA present	■ Ensure that the input cfDNA into the library prep is free of high molecular weight genomic DNA. ■ If needed to salvage a sample, it may be possible to remove the high molecular weight genomic DNA from the input cfDNA by choosing an appropriate size selection method.
	Contaminating DNA present	■ Run a negative control (for example, water) to ensure that there is no product from adapter-only samples.
No libraries present on the Bioanalyzer after the final PCR	Bioanalyzer did not run properly	■ Ensure that the Bioanalyzer is operating properly. Re-run the sample.
	Sample loss	■ Ensure that the DNA was not accidentally discarded during the enrichment procedure. ■ The final PCR step must include the streptavidin beads, which have the enriched library bound to it.
Low yield of the pre-enrichment library	Poor ligation efficiency	■ Ensure that the proper amount of input DNA and Sample Adapter is used. ■ Ensure proper ligation incubation time and temperature are used.
	Poor PCR amplification	■ High adapter dimers (visible near 150 bp) can inhibit the PCR reaction. Follow proper cleanup steps before PCR. ■ Ensure that the first PCR reaction is set up properly.
	Sample loss	■ Ensure that bead cleanup steps are performed properly.
Low yield of the final enriched library	Poor binding with the streptavidin beads	■ Ensure that the proper beads (M-270) were used. ■ Ensure thorough washing and preparation of the M-270 beads. by carefully following the procedure "Preparing the streptavidin beads"
	Sample loss	■ Ensure that the streptavidin beads containing bound libraries are not accidentally discarded during the enrichment procedure. ■ Ensure that the final PCR step includes the streptavidin beads, which have the enriched library bound to them. ■ Ensure that bead cleanup steps are performed properly.
	Poor PCR amplification	■ Ensure that the final PCR reaction is set up properly.
High PCR yield from the final enriched library	Poor on-target rate	■ See *Poor on-target rate on the Sample Metrics Report* in *Post-sequencing QC troubleshooting*.

Issue	Cause	Possible Resolution
High adapter dimers in the final enriched library	Poor hybridization washing	■ Ensure thorough washing by carefully following the procedure *Performing the hybridization cleanup*. ■ Ensure that the correct tube manufacturer is used and that fresh tubes are used when indicated in the protocol.
	Poor PCR clean-up	■ Ensure proper volumes are used in the reaction. ■ Clean the sample again, maintaining the sample to beads ratio. Note that this additional cleanup may result in some sample loss and lower Unique Depth.

Post-sequencing QC troubleshooting

Issue	Cause	Possible Resolution
Poor on-target rate on the Sample Metrics Report	Wrong Enhancing Oligo used	■ Ensure that the proper Enhancing Oligo was used during the hybridization. Check to see if the Enhancing Oligo number matches the Sample Adapter number used during the ligation step.
	Poor washing of the streptavidin beads post-hybridization	■ Ensure thorough washing by carefully following the procedure *Preparing the streptavidin beads*. ■ Ensure that the correct tube manufacturer is used and that fresh tubes are used when indicated in the protocol.
	Improper temperature for incubation	■ Ensure that hybridization occurred at the correct temperature, and work swiftly during the washing procedure post-hybridization.
Low "Number of Read Pairs" on the Sample Metrics Report	Poor sequencing run	■ Refer to an up-to-date Illumina manual. ■ Ensure that samples are free of adapter dimers through Bioanalyzer of the sample.
Low "Median Unique Depth" on the Sample Metrics Report	Poor sequencing run	■ Refer to an up-to-date Illumina manual. ■ Ensure that samples are free of adapter dimers through Bioanalyzer of the sample.
	Poor on-target rate	■ See *Poor on-target rate on the Sample Metrics Report*.
	Poor ligation efficiency	■ Use recommended input amount and proper Sample Adapter volume.
Long "Fragment Length" in the Sample Metrics Report	Presence of high molecular weight DNA	■ See *High molecular weight DNA present in isolated cfDNA.*
Low "Percent Mapped Reads" in the Sample Metrics Report	Presence of non-human samples contaminating the sample	■ Ensure good lab practices when processing the sample. ■ Maintain good Illumina NextSeq 500/550/550Dx maintenance. ■ Perform a manual post-run wash according to Illumina's manufacturer protocols before resequencing.
Low reads "Aligned to PhiX" in the Sample Metrics Report	Not enough PhiX loaded with the samples	■ Ensure proper storage and handling of PhiX. ■ Ensure proper quantification of PhiX concentration. Add extra PhiX, if needed. ■ Refer to an up-to-date Illumina manual.